Supplementary Material

PGC-1α, A Potential Therapeutic Target for Early Intervention in Parkinson's Disease

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1. Supplementary Methods

Brain samples for Middleton-1, Middleton-2, and Miller GWES. Postmortem brain tissue was obtained from the substantia nigra (SN) and the globus pallidus interna (GPi) in two groups of subjects either diagnosed with neuropathologically confirmed PD or pathologically normal controls with no history of major brain illness. Cases with any additional neuropathological diagnoses were excluded. The tissue blocks containing these regions were acquired from the University of Maryland Brain and Tissue Bank, the New York Brain Bank at Columbia University (AG08702-15 and NS16367-24), the Human Brain and Spinal Fluid Resource Center at the West Los Angeles VA Medical Center (sponsored by NINDS/NIMH, the National Multiple Sclerosis Society and the Veterans Health Services and Research Administration), McLean Hospital of Harvard University (MH068855), and the Miami Brain and Tissue Bank (NICHD contract #NO1-HD-8-3284). Postmortem intervals (PMI) were all 24 hr or less. Since agonal state can greatly influence the RNA expression profile of postmortem brain tissue, care was taken to match subject groups as closely as possible for age, sex, PMI, and brain pH. There were no significant differences in these variables between control and PD groups ($P > 0.05$). For the analyses of GPi, gross dissection of the gray matter within each structure was performed on a dry ice block using a dissecting microscope and sterile scalpel. These experiments were conducted in accordance with the Institutional Review Board of SUNY Upstate Medical University. For the Miller GWES, total RNA was extracted from substantia nigra of eight individuals with PD and nine neuropathologically normal controls from the Human Brain and Spinal Fluid Resource Center, VAMC, Los Angeles, CA, the Mind Unit Brain Bank at the University of Rochester, Rochester, NY, and the University of Rochester Alzheimer ’s Disease Center brain bank. This protocol was reviewed by the Human Subjects Review Board at the University of Rochester.

Lymphoblastoid cell lines. All lymphoblastoid cell lines used for the Middleton-3 GWES were obtained from the NINDS Coriell Cell Repository. These cell lines (from age-and sex-matched Parkinson and control subjects, ages 68-73) were established by Epstein-Barr Virus transformation of peripheral B lymphocytes with phytohemagluttinin as a mitogen. After receipt, cells were passaged once for 24 hr in 10 ml RPMI 1640 media using T25 flasks at 37°C with 5% CO2. Cells were pelleted and total RNA isolated from approximately one million cells. These experiments were conducted in accordance with the Institutional Review Board of SUNY Upstate Medical University.

GWES. For the Middleton-1, -2, -3 GWES total RNA was extracted from laser-captured SNpc dopamine neurons, GPi, or human lymphoblastoid cells (HLC) and analyzed by Agilent Bioanalyzer. All RNA samples used in these analyses showed a 28S:18S rRNA ratio > 1.0, and had no evidence of marked RNA degradation. Amplification and labeling of the RNA from laser-captured SNpc dopamine neurons and GPi samples, respectively, were performed using the WT-Ovation™ RNA Amplification System (NuGen, San Carlos, CA). RNA samples from HLCs were prepared using One Cycle Target Labeling Protocol (Affymetrix). Processing of the U133 2.0 GeneChips was performed according to standard protocol (GeneChip Expression Analysis Technical Manual 701021 rev 5, Affymetrix, Santa Clara, CA). After scanning, the microarray images were analyzed using GeneChip Operating System (GCOS) software to obtain performance metrics. For the Miller GWES cRNA was synthesized, hybridized to Affymetrix U133A arrays, and scanned according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction. For the two qPCR validation studies (**Fig. 4**), 19 nuclear-encoded ETC genes (including ten *PGC1*α-responsive genes) were selected based on the stage 2 microarray analysis, TaqMan Assay-on-demand primers and probes were designed according to the manufacturer's guidelines including crossing of exon junctions and arrayed on 384-well microfluidic cards. RNA was extracted, cDNA was synthetized as described (*S1*), and qPCR was performed on an ABI 79000HT and analyzed by comparative threshold cycle method (*S2*) using Ubiquitin C (*UBC)* as reference gene. Similar results were obtained when the ribosomal gene *RPL13* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used to control for input RNA. Control reactions lacking template or reverse transcription showed no amplification. Target and reference genes showed similar amplification efficiencies in a dilution series. Primer/probe information is available upon request.

For qPCR on cultured rat primary midbrain neurons (**Fig. 5**), qPCR was performed similar to above but the TaqMan Gene Expression Cells-to-CT Kit (Applied Biosystems, Carlsbad, California) was used to synthesize cDNA and rodent-specific TaqMan Assay-on-demand primers and probes were used to assay relative abundance of target genes; rodent *RPL13* was used as endogenous control; primary midbrain cultures transduced with A53T-α-synuclein alone served as calibrator. Similar results were obtained when *UBC* was used to control for input RNA. For qPCR on cultured, rotenone-treated SH-SY5Y cells (**Fig. 6**) RNA was extracted and cDNA was synthesized as we described (*S1*). Nuclear-encoded ETC target genes were analyzed using *RPL13* to control for input RNA and using cells treated with rotenone alone as calibrator. Again, similar results were obtained when *UBC* was used to control for input RNA.

Preparation of adenoviral constructs. A53T-α-synuclein and *PGC-1α* were expressed in primary midbrain cultures from adenoviruses generated with the ViraPower Adenoviral Expression System (Invitrogen). The preparation of an adenoviral construct encoding human A53T-α-synuclein was described previously (*S3*). A cDNA encoding human *PGC-1α* was amplified from the construct pcDNA4-myc-*PGC-1α* by PCR and subcloned into the Kpn I and Xho I sites of the vector pENTR1A to generate pENTR-*PGC-1α.* The insert from pENTR-*PGC-1α* was transferred into the pAd/CMV/V5 DEST adenoviral expression vector via a recombination reaction and sequenced using an Applied Biosystems DNA sequencer at Purdue University.

Preparation of primary mesencephalic cultures. Primary midbrain cultures were prepared via dissection of day 17 embryos obtained from pregnant Sprague-Dawley rats (Harlan) as described (*S4*). All of the procedures involving animal handling were approved by the Purdue Animal Care and Use Committee. The cells were plated on coverslips at a density of 1250 cells per mm^2. Five days after plating, the cells were treated with AraC (20 μM, 48 h) to inhibit the growth of glial cells. At this stage (i.e. 7 days in vitro), the glial cells accounted for approximately 50% of the total cell population, and the neurons appeared differentiated with extended processes.

Adenoviral transductions and immunocytochemical analysis of primary neurons. For experiments aimed at determining the effects of *PGC-1α* over-expression on A53T-α-synuclein neurotoxicity, primary cultures (7 days in vitro) were untransduced or transduced with adenovirus for 72 h at a multiplicity of infection (MOI) of 4 (*PGC-1α* virus) or 10 (A53T virus) (*S3, S4*). The cells were then treated with fresh media for an additional 24 h and

stained for microtubule associated protein 2 (MAP2) and tyrosine hydroxylase (TH) as described (*S3*, *S4*). For experiments aimed at determining the effects of *PGC-1α* over-expression on rotenone neurotoxicity, primary cultures (7 days in vitro) were untransduced or transduced with *PGC-1α* adenovirus (MOI = 4) for 72 h and then treated with fresh media supplemented with rotenone (100 nM) for 24 h prior to staining for MAP2 and TH.

Measurement of primary neuron viability. MAP2- and TH-immunoreactive primary neurons were counted in 10 randomly chosen observation fields for each experimental condition using a Nikon TE2000-U inverted fluorescence microscope with a 20x objective. Typically, 300-1500 $MAP2^+$ neurons were counted per experiment for each condition, a range that corresponds to 10-60 TH^+ neurons. The data were expressed as the percentage of $MAP2^+$ neurons that were also TH^+ (this ratiometric approach was used to correct for variations in cell density). Each experiment was repeated 3 or 4 times with primary cultures prepared from independent pregnant rats. Statistical analyses were carried out with the program GraphPad Prism, Version 4.0. Statistical significance was verified by one-way ANOVA with the Newman-Keuls post-test.

SH-SY5Y cell culture, adenoviral transductions, and rotenone treatment. SH-SY5Y human neuroblastoma cells from American Type Culture Company (ATCC, Rockville, MD) were cultured in DMEM/F12 (Invitrogen, Carsbad, CA) supplemented with 10% fetal bovine serum and 50 U/ml penicillin/streptomycin. Cultures were maintained at 37°C with 95% humidified air and 5% CO_2. SH-SY5Y cultures were untransduced or transduced with adenovirus encoding *PGC1α* or LacZ (MOI of each virus = 50) for 24 h (*S3, S4*). Then rotenone (Sigma, St. Louis, MO) at a final concentration of 10 μM was added for 24 hours.

For each experiment, rotenone was prepared fresh in dimethylsulfoxide (DMSO). The DMSO concentration in the medium did not exceed 0.1% (v/v).

MTT cell viability assay. SH-SY5Y cells were plated at a density of $4x10^4$/well in a 96-well plate. 15µl of MTT (Promega, Madison, WI) was added to each well and the cells were incubated at 37 °C for 4 hours. After incubation, 100 µl of solubilization/stop solution was added to each well. The absorbance at 570 nm was measured with a microplate reader 1 hour later. Statistical analysis was carried out by InStat 3.0 using a two-sided t-test.

2. Supplementary Tables

Table S1 Stage 1 meta-analysis results with the outlier data set removed

			Stage 1 (DA & SN)	
Gene set	N Genes	Annotation	sNES	*P* value#
Electron transport chain	95	Broad	-1.665	$< 1x10^{-6}$
MAP00190 Oxidative phosphorylation	46	GenMAPP	-1.672	$< 1x10^{-6}$
MAP00620 Pyruvate metabolism	31	GenMAPP	-1.542	$< 1x10^{-6}$
VOXPHOS	87	BioCarta	-1.612	$< 1x10^{-6}$
Mitochondr	447	Broad	-1.509	$< 1x10^{-6}$
Krebs-TCA Cycle	29	BioCarta	-1.487	$3.42x10^{-6}$
Human mitoDB 6 2002	428	Broad	-1.476	$< 1x10^{-6}$
GO 0005739	170	GO	-1.444	$< 1x10^{-6}$
PGC	425	Broad	-1.427	$2.05x10^{-5}$
ChREBP pathway	20	Broad	-1.376	$3.41x10^{-5}$
Urea cycle pathway	7	KEGG	-1.332	$6.81x10^{-5}$
MAP00252 Alanine and aspartate metabolism	21	GenMAPP	-1.224	0.0010249

DA, GWES of laser-captured SN dopamine neurons; SN, substantia nigra GWES; subclinical PD-LBN, GWES of SN from individuals with subclinical, mild, PD-related Lewy body neuropathology; # *P* values here were estimated based on 1 million permutations (compared to 100 million permutations used for Table 2).

Table S2 Twenty-eight gene sets associated with PD in stage 1.

			Stage I (DA & SN)		Stage II (Subclinical PD-LBN)		Stage III (non-SN)		All data			All SN data		
Gene set #	Genes	Source #	sNES	*P* value	NES	*P* value	sNES	*P* value	N	sNES	*P* value	N	sNES	*P* value
Electron transport chain	95	Broad	-1.583	$<1x10^{-8}$	-1.496	$1.46x10^{-2}$	-1.420	$1.66x10^{-5}$	410	-1.519	$<1x10^{-8}$	218	-1.580	$<1x10^{-8}$
MAP00190 Oxidative phosphorylation	46	GenMAPP	-1.572	$<1x10^{-8}$	-1.716	$4.70x10^{-2}$	-1.132	$2.52x10^{-3}$	410	-1.388	$<1x10^{-8}$	218	-1.586	$<1x10^{-8}$
MAP00620 Pyruvate metabolism	31	GenMAPP	-1.529	$3.36x10^{-8}$	-1.844	$2.37x10^{-2}$	-1.062	$4.59x10^{-3}$	410	-1.332	$<1x10^{-8}$	218	-1.541	$<1x10^{-8}$
VOXPHOS	87	BioCarta	-1.527	$1.34x10^{-7}$	-1.451	$2.28x10^{-2}$	-1.389	$1.94x10^{-5}$	410	-1.471	$<1x10^{-8}$	218	-1.524	$7.92x10^{-8}$
Mitochondr	447	Broad	-1.464	$6.76x10^{-7}$	-1.761	$1.43x10^{-2}$	-1.247	$5.21x10^{-4}$	410	-1.376	$<1x10^{-8}$	218	-1.479	$5.54x10^{-7}$
Krebs- TCA Cycle	29	BioCarta	-1.447	$3.38x10^{-7}$	-1.633	$3.02x10^{-2}$	-1.184	$1.28x10^{-3}$	410	-1.359	$6.22x10^{-8}$	218	-1.462	$8.71x10^{-7}$
Human mitoDB 6 2002	428	Broad	-1.427	$3.38x10^{-7}$	-1.750	$1.23x10^{-2}$	-1.271	$4.21x10^{-4}$	410	-1.373	$<1x10^{-8}$	218	-1.445	$5.32x10^{-7}$
GO 0005739	170	GO	-1.369	$3.72x10^{-6}$	-1.758	$2.04x10^{-2}$	-1.230	$5.87x10^{-4}$	410	-1.322	$3.11x10^{-8}$	218	-1.391	$3.19x10^{-6}$
PGC	425	Broad	-1.366	$6.75x10^{-6}$	-1.576	$4.96x10^{-2}$	-0.884	$1.65x10^{-2}$	410	-1.165	$6.32x10^{-7}$	218	-1.379	$2.93x10^{-6}$
ChREBP pathway	20	Broad	-1.280	$3.34x10^{-5}$	-2.100	$1.19x10^{-2}$	-0.799	$3.38x10^{-2}$	410	-1.127	$3.16x10^{-7}$	218	-1.341	$6.92x10^{-6}$
Ureacycle pathway	7	KEGG	-1.262	$6.77x10^{-5}$	-1.671	$1.46x10^{-2}$	-0.575	$1.15x10^{-1}$	410	-0.994	$2.21x10^{-5}$	218	-1.294	$2.65x10^{-5}$
MAP00252 Alanine and aspartate metabolism	21	GenMAPP	-1.165	$3.39x10^{-5}$	-1.831	$1.80x10^{-2}$	-0.482	$1.91x10^{-1}$	410	-0.908	$1.58x10^{-4}$	218	-1.213	$1.87x10^{-4}$
Mitochondria pathway *	21	BioCarta	1.319	$4.27x10^{-5}$	-1.669	$4.78x10^{-2}$								
TCA	15	Broad	-1.473	$2.69x10^{-7}$	-1.476	$5.26x10^{-2}$								
MAP00251 Glutamate metabolism	21	GenMAPP	-1.277	$6.75x10^{-5}$	-1.617	$5.34x10^{-2}$								
Sodd pathway	10	BioCarta	1.281	$8.49x10^{-5}$	1.725	$5.42x10^{-2}$								
Etc pathway	10	BioCarta	-1.353	$1.02x10^{-5}$	-1.396	$6.49x10^{-2}$								
MAP00650 Butanoate metabolism	21	BioCarta	-1.381	$3.39x10^{-5}$	-1.516	$6.84x10^{-2}$								
MAP00020 Citrate cycle TCA cycle	18	GenMAPP	-1.480	$3.38x10^{-7}$	-1.527	$7.32x10^{-2}$								
Vif pathway	3	BioCarta	1.323	$4.25x10^{-5}$	1.445	$1.13x10^{-1}$								
MAP00193 ATP synthesis	19	GenMAPP	-1.433	$1.69x10^{-6}$	-1.491	$1.17x10^{-1}$								
MAP 03070 Type III secretion system	19	GenMAPP	-1.435	$1.35x10^{-6}$	-1.491	$1.17x10^{-1}$								
MAP00195 Photosynthesis	20	GenMAPP	-1.421	$3.39x10^{-6}$	-1.543	$1.21x10^{-1}$								
Kreb pathway	8	BioCarta	-1.421	$1.35x10^{-6}$	-1.338	$1.67x10^{-1}$								
MAP00010 Glycolysis and gluconeogenesis	55	GenMAPP	-1.368	$1.01x10^{-5}$	-1.496	$1.84x10^{-1}$								
MAP00630 Glyoxylate and dicarboxylate metabolism	10	GenMAPP	-1.370	$6.77x10^{-6}$	-1.317	$2.04x10^{-1}$								
Death pathway	33	BioCarta	1.419	$5.68x10^{-7}$	-1.331	$2.05x10^{-1}$								
MAP00720 Reductive carboxylate cycle CO2 fixation	7	BioCarta	-1.527	$6.72x10^{-8}$	-1.207	$3.17x10^{-1}$								

DA, GWES of laser-captured SN dopamine neurons; SN, substantia nigra GWES; subclinical PD-LBN, GWES of SN from individuals with subclinical, mild, PD-related Lewy body neuropathology;
* Note that the direction of change of this gene set was not replicated in stage II (positive sNES in stage I, but negative NES in stage II); # gene set sources and gene set nomenclature correspond to version 1.1 of the MSigDB C2.

Table S3. Clinicopathologic characteristics of SN specimens used in the stage 2 analysis. 16 Cases with subclinical, mild PD-related Lewy body neuropathology (incidental Lewy body disease) and 17 age-, sex-, PMI-matched controls were analyzed in the stage 2 validation study.

Pathological diagnosis	Age	Gender	PMI	Lewy bodies in brainstem[$] or olfactory bulb**	Lewy bodies in neocortex***	Clinical diagnosis
Control	73	F	1.5	-	-	Control
Control	85	F	2.75	-	-	Control
Control	87	F	2.83	-	-	Control, essential tremor
Control	86	F	2.5	-	-	Control
Control	86	F	1.5	-	-	Control
Control	78	F	3.75	-	-	Control
Control	56	F	11.83	-	-	Control
Control	45	F	33.8	-	-	Control
Control	80	M	2.16	-	-	Control, tremor disorder
Control	78	M	1.66	-	-	Control
Control	83	M	3.16	-	-	Control
Control	91	M	1.5	-	-	Control
Control	89	M	2.5	-	-	Control
Control	95	M	3.5	-	-	Control
Control	60	M	26.15	-	-	Control
Control	58	M	17.4	-	-	Control
Control	40	M	28	-	-	Control
ILB*	89	F	26	+	-	Control
ILB	83	F	2.5	+	-	Control, depressive pseudodementia
ILB	78	F	3.33	+	#	Control
ILB	88	F	3.5	+	-	Control
ILB	94	F	2.5	+	-	Control
ILB	82	F	2.5	+	-	Control, acute myeloid leukemia
ILB	87	F	2	+	-	Control
ILB	103	F	3	+	#	Mild cognitive impairment
ILB	56	M	17.3	+	-	Control
ILB	57	M	21	+	-	Control
ILB	57	M	21.25	+	-	Control
ILB	85	M	3.16	+	-	Control
ILB	92	M	3.83	+	-	Control
ILB	90	M	2.83	+	-	Control
ILB	86	M	2.8	+	-	Control, tremor disorder
ILB	94	M	3.5	+	-	Cognitively normal, multiple stokes, tremor disorder

*ILB, Incidental Lewy body disease; ** five individuals had Lewy bodies in the olfactory bulb only; [$] brainstem regions included locus coeruleus and substantia nigra; *** neocortical regions examined in most brains: midfrontal and midtemporal gyrus, inferior parietal lobe; [#] these subjects had Lewy bodies in the transenthorinal cortex

3. Supplementary Figures

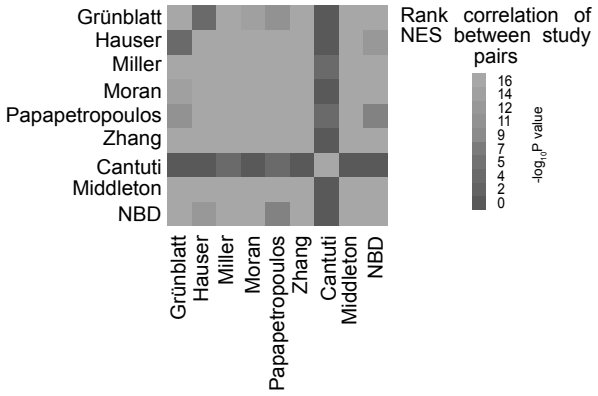

Figure S1. **NESs of 522 gene sets correlate between independent studies.** Plot of *P* values of Spearman correlations between any pair of the nine data sets. *P*-values are color-coded with light gray indicating highly significantly correlated NES between study pairs and dark gray indicating no correlation. NESs are significantly correlated between eight of nine studies (*P* values ranging from 0.002 to 10^{-16}) indicting that gene set enrichment is replicated in genome-wide expression data from independent biological samples (independent patients), from distinct RNA sources (laser-captured SN dopamine neurons, SN homogenates), on distinct platforms (one Affymetrix Focus array, four Affymetrix U133A arrays, two Affymetrix U133 Plus 2.0 arrays, and one Affymetrix U133 Set). Note that NESs of one outlier data set (Cantuti) do not correlate with the other eight studies (*P* values 0.0001 ~ 0.62) potentially due to the non-standard probes on the Affymetrix U133_X3P array used by ref. (*S5*), which differ from the probes on the other Affymetrix arrays included in the meta-analysis because of a 3' end bias. On the X3P arrays the majority of probes are selected from the 300 bases at the most 3' end of the transcripts. This is different from the standard Affymetrix design strategy, which selects probes within the region of 600 bases proximal to the 3' ends.

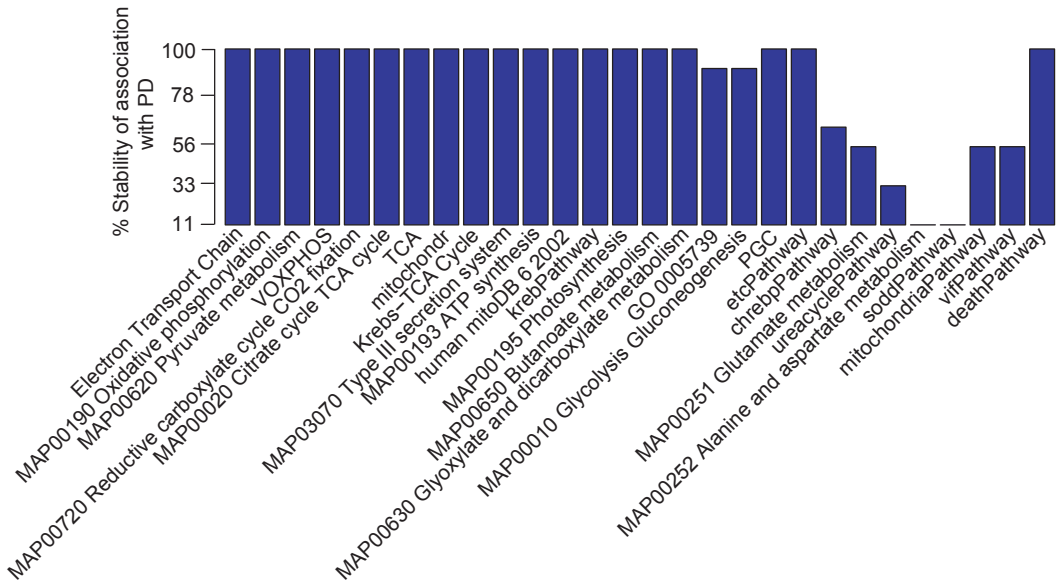

Figure S2. **Stability of pathway associations identified by meta-GSEA.** The x-axis represents the 28 significant gene sets identified in the stage 1 meta-GSEA sorted by their summary-NES. The y-axis represents stability of the association with PD. We iteratively left one study out at a time, performed meta-GSEA for the remaining eight studies, and scored the number of times one of the 28 gene sets achieved genome-wide significance ($P < 9.6 \times 10^{-5}$ in each of nine unique iterations) in the left-in studies. Stability was 100% for 19 of the 28 gene sets, while the results for remaining gene sets were considerably less stable.

Supplemental References

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